








Clinical Validation of an Immune Quiescence Gene Expression Signature in Kidney Transplantation

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Key Points

- AlloMap Kidney is a gene expression profile developed using candidate genes from the AlloMap assay broadly used in heart transplantation.
- AlloMap Kidney was validated to differentiate quiescence from rejection in two independent sample sets using a quantitative scale.
- Blood cell gene expression and donor-derived cell-free DNA contribute independent signals and inform on different aspects of allograft rejection.

Abstract

Background Despite advances in immune suppression, kidney allograft rejection and other injuries remain a significant clinical concern, particularly with regards to long-term allograft survival. Evaluation of immune activity can provide information about rejection status and help guide interventions to extend allograft life. Here, we describe the validation of a blood gene expression classifier developed to differentiate immune quiescence from both T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR).

Methods A five-gene classifier (DCAF12, MARCH8, FLT3, IL1R2, and PDCD1) was developed on 56 peripheral blood samples and validated on two sample sets independent of the training cohort. The primary validation set comprised 98 quiescence samples and 18 rejection samples: seven TCMR, ten ABMR, and one mixed rejection. The second validation set included eight quiescence and 11 rejection samples: seven TCMR, two ABMR, and two mixed rejection. AlloSure donor-derived cell-free DNA (dd-cfDNA) was also evaluated.

Results AlloMap Kidney classifier scores in the primary validation set differed significantly between quiescence (median, 9.49; IQR, 7.68–11.53) and rejection (median, 13.09; IQR, 11.25–15.28), with $P < 0.001$. In the second validation set, the cohorts were statistically different ($P = 0.03$) and the medians were similar to the primary validation set. The AUC for discriminating rejection from quiescence was 0.786 for the primary validation and 0.800 for the second validation. AlloMap Kidney results were not significantly correlated with AlloSure, although both were elevated in rejection. The ability to discriminate rejection from quiescence was improved when AlloSure and AlloMap Kidney were used together (AUC, 0.894).

Conclusion Validation of AlloMap Kidney demonstrated the ability to differentiate between rejection and immune quiescence using a range of scores. The diagnostic performance suggests that assessment of the mechanisms of immunologic activity is complementary to allograft injury information derived from AlloSure dd-cfDNA. Together, these biomarkers offer a more comprehensive assessment of allograft health and immune quiescence.

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Introduction

Despite modern immune suppression regimens, kidney allograft rejection continues to be both a common occurrence and the primary driver of unacceptably high graft failure rates (1–3). Ten percent of kidney transplant recipients experience allograft rejection in the first year after transplant (4). Although allograft biopsy is the current standard for diagnosis of rejection, optimizing the appropriateness of biopsies by noninvasive techniques is imperative due to the invasive nature of the procedure and the associated risk, and the sampling error and subjective nature of histopathologic interpretation. Analysis of a large series of renal transplant protocol biopsy specimens demonstrated a 2% major complication rate and a 5% risk of gross hematuria (5,6). Additionally, up to 15% of biopsies yield an inadequate specimen, exposing patients to procedural risk without diagnostic benefit (7). The ability to enhance the timing and diagnostic yield of biopsies could meaningfully improve post-transplant outcomes. In addition, methods for assessing response to rejection treatment and return to baseline allograft function frequently rely on additional follow-up biopsies, all associated with risks, expense, inconvenience, and diagnostic failure.

Serum creatinine is commonly used to assess kidney function as a screen for rejection. However, allograft damage sufficient to impair renal function is often irreversible (8), and serum creatinine has repeatedly been shown to be a poorly sensitive or specific indicator of rejection (9). Robust diagnostic and prognostic biomarkers that provide evidence of graft rejection ahead of pathologic findings are needed to help guide clinical management of transplant patients. Among the best studied advanced biomarkers in transplantation is the plasma level of donor-derived cell-free DNA (dd-cfDNA) used to assess allograft injury (9–12). dd-cfDNA has gained significant adoption since it became available as a clinically validated test (12), with numerous studies demonstrating clinical utility in a broad array of contexts (10,11,13–15).

Additional assays are those that use gene expression of circulating immune cells to evaluate immune activity. One example of a broadly integrated gene expression profile (GEP) assay is AlloMap, available as a surveillance tool for recipients of heart transplants since 2005 (16). The assay methodology has not changed since validation and subsequent publication of clinical utility (17); the high negative predictive value (NPV) has enabled avoidance of biopsies for >15 years by the heart transplant community (18). Gene expression profiling of the immune system in kidney transplantation, however, has been elusive as a consistently reliable and reproducible measure of rejection. Several plasma gene expression panels have been published (19–22), one of which is commercially available and designed for use specifically in place of the protocol biopsy (19). Another has faced concerns after independent studies were unable to replicate the validation (20,23). Other signatures with a strong association with fibrosis (21) or specific to antibody-mediated rejection (ABMR) (22) have not yet achieved routine clinical use.

A focused determination of the immune state as active compared with quiescent can help to assess the likelihood of allograft rejection. We describe validation of a blood GEP that can stratify samples according to likelihood of

immune quiescence versus T cell-mediated rejection (TCMR) or ABMR. Building on the extensively demonstrated utility of the AlloMap gene set (17), we developed a novel classifier for kidney allograft rejection, condensing the 11 informative genes from AlloMap to a five-gene subset. The AlloMap Kidney classifier was validated using two sample sets independent of the training set, and the performance was evaluated in the primary validation both independently and in conjunction with AlloSure dd-cfDNA.

Materials And Methods

Study Design

Three sets of data were used in this study. The training set and the primary validation set consisted of randomly assigned (per cohort), distinct sets of patients from the DART study (ClinicalTrials.gov, NCT02424227), a multicenter, prospective, observational study to collect plasma in Streck Cell-Free DNA BCT for the purpose of dd-cfDNA measurement and whole blood RNA in PAXgene tubes for gene expression profiling (9). The institutional review board (IRB) at each site approved the study and all of the patients provided written informed consent. The study sponsor provided the statistical analysis, data management, and clinical operations coordination. A second validation set was used to further validate the performance of AlloMap Kidney. These samples, from Montefiore Medical Center (Bronx, NY), were from patients not included in the DART study. Samples were derived from an IRB-approved study of “immune monitoring of kidney transplant recipients” (IRB number 09-06-174). The clinical and research activities reported are consistent with the Principles of the Declaration of Istanbul, as outlined in the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

Patients and Samples

Patient samples were assigned to two general cohorts: Rejection or Quiescence. Each cohort was defined by established Banff criteria (24), and each contained subgroups as follows. The rejection samples comprised TCMR, ABMR, or mixed rejection (meeting criteria for both ABMR and TCMR). The quiescence samples were one of three types: healthy stable (HS), which had no clinical or laboratory indicators of concern for the graft (and, therefore, no clinically indicated biopsy) and a low level of dd-cfDNA, as measured by AlloSure (<0.5%); nonrejection (NR), which were determined to not have signs of rejection upon pathologist review after a clinically indicated biopsy; and protocol NR (pNR), which were determined to not have signs of rejection upon pathologist review after a protocol surveillance biopsy. Because it is standard to include the best-defined members of the two main cohorts when training a classifier, the Rejection sets for training and the primary validation set included TCMRIA, IB, IIA, and IIB along with ABMR. Borderline TCMR samples were a part of the rejection group in the second validation set. For ABMR, acute/active and chronic, active ABMR were included.

RNA Purification

The DART PAXgene blood tubes were collected alongside Streck BCT plasma, shipped at ambient temperature,

received within 3 days, and stored at -80°C until RNA extraction. After thawing, RNA was purified using the QIASymphony PAXgene Blood RNA Kit (catalog number, 762635; Qiagen) on the QIASymphony SP system, following the manufacturer's instructions. The second validation set samples were extracted manually using PAXgene Blood RNA Kits (catalog number, 762164; Qiagen) according to the manufacturer's instructions. The concentration and purity of the extracted RNA samples were determined by spectrophotometry. Samples were also analyzed for integrity by capillary electrophoresis (TapeStation; Agilent).

Quantitative RT-PCR Methodology

The purified RNA samples were subjected to quantitative RT-PCR (qRT-PCR) in the CareDx CLIA laboratory as described (16). The qPCR for each gene was run in triplicate, and the raw threshold cycle (C_T) values (at which probe fluorescence reaches the measurement threshold) were used to calculate a smoothed mean C_T . The mean C_T was then used for the development of the AlloMap Kidney signature, as described below, without using additional data processing methods or procedures from AlloMap Heart.

Classifier Training

The mean C_T for the candidate test genes was normalized against six reference genes (Supplemental Table 2), which were selected on the basis of their stability in this sample set using a scheme similar to what was described previously (16). The normalized results were assessed for statistical significance in a univariate model. Six genes identified as statistically significant were then crossvalidated *via* bootstrapping and leave-one-out validations. The five genes that passed these internal validations were grouped into three clusters on the basis of their normalized C_T level across the full set of training samples. Each cluster has a pairwise correlation coefficient >0.6 . A multivariate model that integrates the normalized expression of the five genes was built to optimize performance to differentiate rejection from quiescence in the training sample set.

RNA-Sequencing Methodology

RNA sequencing (RNA-seq) was chosen as a validation and testing platform to enable improved detection of low-expression genes, higher reproducibility, and accurate measurement of gene expression changes that can be readily expanded to additional gene sets and classifiers. A targeted RNA-seq panel (QIAseq; Qiagen), which includes the five informative genes, 15 reference genes (Supplemental Tables 1 and 2), genomic DNA contamination controls, and spike-in controls, was developed and optimized for PAXgene blood RNA samples on an RNA-seq platform using molecular tags (25). Single-read sequencing was performed on an Illumina NextSeq 550. Primary analysis of the sequencing data was performed using the Qiagen GeneGlobe QIAseq bioinformatics pipeline for adaptor trimming, read mapping, quality checks, and computing the molecular tag counts (MTs) for the targeted transcripts. Because the MTs are directly correlated with the initial copy number of the input RNA, a conversion could be defined and tested (Supplemental Appendix 1, Supplemental Figures 1 and 2)

to convert MTs to a C_T value that would match the C_T generated on the same sample by qPCR. The corresponding C_T number was derived using the equation $X_0 = E_{\text{amp}}^{(b - C_T)}$, where E_{amp} is the exponential amplification value, b is the y intercept of $\log(\text{copies})$ versus C_T , using the average amplification efficiency of 98%, and $b=39$ for the AlloMap qRT-PCR tests. The C_T values for the informative genes were normalized using the average C_T of the reference genes. The normalized C_T numbers were then used to compute the AlloMap Kidney score using the locked classifier algorithm trained on the qRT-PCR data. AlloSure measurement of dd-cfDNA was performed as previously described (26).

Statistical Analysis

The analysis of differences between groups was performed using an unpaired t test; performance metrics were calculated using standard methods in JMP version 13. Receiver operating characteristic (ROC) curves were generated using the pROC package in R (version 4.0.5). To generate ROC plots for combined AlloMap Kidney and AlloSure data, the AlloSure score was log transformed and then converted to the standard normal distribution. The AlloMap Kidney score was converted to the standard normal distribution similarly. The two converted scores were added arithmetically to create a single result for each sample to plot the ROC.

Results

The AlloMap Heart gene set was developed using PBMCs and comprises genes implicated in diverse immune pathways; therefore, this was chosen as a source of candidate genes for development of a classifier in kidney transplantation (Supplemental Table 1) (16). Due to the complexity of purifying PBMCs at the time of collection, whole blood samples were collected in PAXgene tubes from recipients of kidney transplants. Gene expression data were generated for the 11 AlloMap Heart genes from a subset of the patients from the DART study who were designated as the training sample set, with 38 samples from 22 patients classified as quiescence (HS with AlloSure $<1\%$) and 18 samples from 16 patients classified as rejection (seven TCMR, eight ABMR, three mixed). The only demographic differences between the cohorts in the training sample set were that the Quiescence cohort were earlier post-transplant, and that the Rejection groups had higher serum creatinine and lower eGFR, as expected. No differences were observed in race, sex, type of transplant, recipient or donor cytomegalovirus serology, HLA mismatches, panel-reactive antibodies, induction therapy, or maintenance immunosuppression (Table 1).

Given the differences in sample type and the transplanted organ, a new gene expression classifier was developed starting from the AlloMap Heart gene set. Of the 11 informative genes included in AlloMap Heart (16), six were significantly different between the Quiescence and Rejection cohorts in the training set in a univariate model ($P < 0.02$, Supplementary Table 1). Bootstrap and leave-one-out testing within the training set indicated that five of the six genes were used in $>75\%$ of instances; subsequent stepwise selection yielded three important clusters with five genes. These genes represent biologic functions related to

Table 1. Clinical characteristics of the DART analysis groups

Clinical Characteristic	Training				Validation			P Value (Nonrejection versus Rejection)
	Healthy Stable AlloSure <1%	Rejection	P Value	Nonrejection	Healthy Stable	Protocol Nonrejection	Rejection	
Patients, <i>N</i>	22	16		44	20	19	16	
Samples, <i>N</i>	38	18		47	22	29	18	
Race, <i>n</i> (%)			0.35					0.63
Black	13 (34)	8 (44)		14 (30)	0	8 (28)	4 (22)	
White	21 (55)	9 (50)		25 (53)	15 (68)	19 (66)	13 (72)	
Native	0	1 (6)		0	0	1 (4)	0	
Hispanic/Latino	3 (8)	0		5 (11)	4 (18)	1 (4)	1 (6)	
Asian	0	0		1 (2)	1 (5)	0	0	
Other	1 (3)	0		2 (4)	2 (9)	0	0	
Sex, <i>n</i> (%)			0.88					0.31
Men	24 (63)	11 (61)		30 (64)	14 (64)	21 (72)	9 (50)	
Women	14 (37)	7 (39)		17 (36)	8 (36)	8 (28)	9 (50)	
Age at enrollment, yr, mean±SD	48.8±13.7	47.9±17.8	0.85	50.9±14.5	54.9±11.8	53.8±12.7	39.4±10.9	0.001
Post-transplant, d, mean±SD	94.5±55.3	1091±1071	<0.001	965±1360	244±200	242±192	1322±1213	0.32
CMV serologic status, <i>n</i> (%)			0.05					0.07
D−/R+	10 (26)	3 (17)		13 (28)	7 (32)	12 (41)	3 (17)	
D+/R+	14 (37)	3 (17)		14 (30)	7 (32)	7 (24)	3 (17)	
D−/R−	7 (18)	2 (11)		8 (17)	3 (14)	7 (24)	4 (22)	
D+/R−	4 (10)	3 (17)		5 (11)	2 (9)	3 (10)	0	
Unknown	3 (8)	7 (39)		7 (15)	3 (14)	0	8 (44)	
Donor type, <i>n</i> (%)			0.47					0.12
Deceased	21 (55)	13 (72)		28 (60)	13 (59)	15 (52)	11 (61)	
Living, unrelated	4 (10)	1 (6)		14 (30)	3 (14)	8 (28)	2 (11)	
Living, related	13 (34)	4 (22)		5 (11)	6 (27)	6 (20)	5 (28)	
Creatinine, mg/dl, mean±SD	1.46±0.53	2.47±1.15	<0.001	2.27±1.49	1.36±0.53	1.61±0.57	2.83±0.91	0.08
eGFR, ml/min per 1.73 m ² , mean±SD	55.0±14.9	33.6±13.1	<0.001	38.9±19.8	56.3±19.6	48.2±13.2	25.9±11.3	0.002
HLA class 1, <i>n</i> mismatches (A, B), mean±SD	2.7±1.1	3.1±1.3	0.30	2.5±1.3	2.1±1.1	2.9±1.2	1.7±1.1	0.02
HLA class 2, <i>n</i> mismatches (DR), mean±SD	1.2±0.6	1.2±0.5	0.95	1.1±0.8	1.1±0.8	1.2±0.7	0.9±0.8	0.32
PRA class I (%)			>0.99					0.17
Mean PRA	1.72	1.73		24.1	10.8	9.03	13.4	
Samples	36	15		39	16	29	15	
PRA class II (%)			0.12					0.52
Mean PRA	4.67	18.1		14.8	10.9	2.9	20.9	
Samples	36	14		39	16	29	15	
Induction patients, <i>n</i> (%)			0.05					0.46
ATG	2 (9)	8 (50)		9 (21)	2 (10)	0	5 (31)	
Alemtuzumab	3 (14)	3 (19)		9 (21)	1 (5)	0	2 (13)	
Basiliximab	2 (9)	0		0	1 (5)	0	1 (6)	
Other	3 (14)	3 (19)		8 (18)	2 (10)	0	3 (19)	
None	14 (65)	5 (31)		23 (52)	15 (75)	19 (100)	8 (50)	
Immunosuppression samples, <i>n</i> (%)			0.26					0.52
Cyclosporin	2 (5)	2 (11)		5 (11)	1 (4)	0	2 (11)	
Tacrolimus	35 (92)	16 (89)		38 (81)	20 (20)	27 (93)	15 (83)	
Mycophenolate	34 (90)	14 (78)		40 (85)	20 (91)	28 (97)	15 (83)	
Prednisone	17 (45)	14 (78)		28 (60)	16 (73)	10 (34)	13 (72)	
Rapamycin	2 (5)	1 (6)		3 (6)	0	0	2 (11)	
Azathioprine	2 (5)	1 (6)		3 (6)	0	1 (3)	1 (6)	
Belatacept	1 (3)	0		2 (4)	0	2 (7)	0	
Other	0	3 (17)		0	0	0	2 (11)	

CMV, cytomegalovirus; D−/R+, donor negative/recipient positive; PRA, panel-reactive antibody; ATG, antithymocyte globulin.

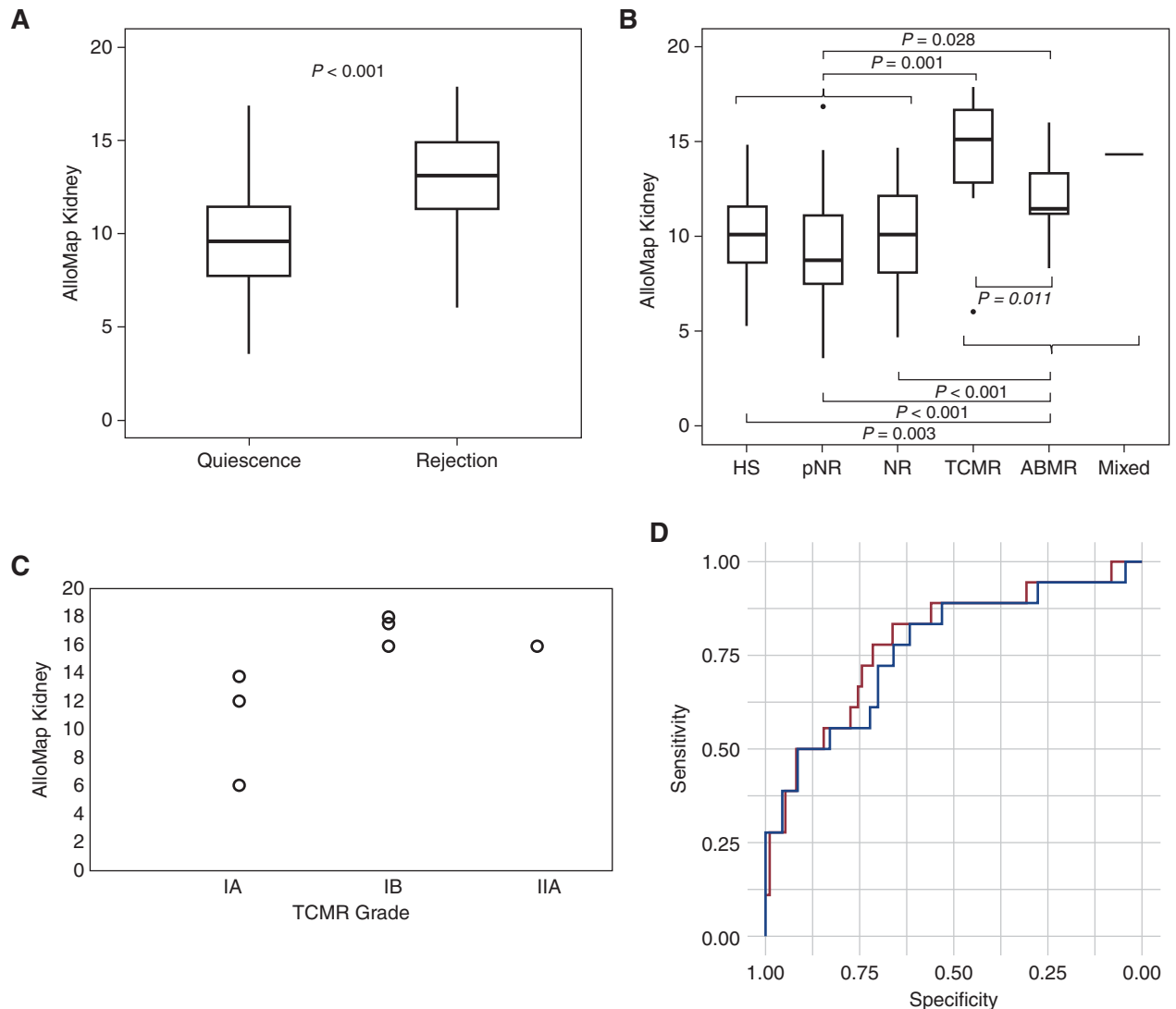


Figure 1. | AlloMap Kidney classifier differentiates quiescence from rejection in the primary independent validation set. (A) Box-and-whisker plots show that biopsy specimen–defined rejections ($n=18$) were significantly different from quiescence ($n=98$). (B) No difference among quiescence subgroups, including nonrejection (NR), protocol NR (pNR), and healthy stable (HS) subgroups; all three were significantly lower than T cell–mediated rejection (TCMR) and antibody mediated rejection (ABMR). HS, $n=22$; pNR, $n=29$; NR, $n=47$; TCMR, $n=7$; ABMR, $n=10$; mixed rejection, $n=1$. (C) TCMR results stratified by grade suggest a trend for AlloMap Kidney and TCMR grade. (D) Receiver operating characteristic plot for the primary validation set with rejection compared with quiescence (red line) or NR (blue line). Unpaired t test.

immune response pathways: DCAF12 and MARCH8 are involved in modulating immune reactivity, FLT3 and IL1R2 are steroid-responsive genes, and PDCD1 is expressed on activated T lymphocytes (Supplemental Table 1) (27). In the training set, the AlloMap Kidney classifier readily distinguished rejection from quiescence, with $P < 0.001$ and with an area under the ROC curve (AUC) of 0.939 (95% CI, 0.89 to 0.99).

A second set of DART samples from patients not contributing to the training sample set was used as the primary validation set. These 99 unique patients contributed 98 quiescence samples (22 HS, 29 pNR, and 47 NR) and 18 rejection samples (seven TCMR, ten ABMR, and one mixed rejection) (Table 1). The only demographic differences between the cohorts in the primary validation set were that

the rejection cohort was younger and had fewer HLA class 1 mismatches. There is not a statistical difference in time post-transplant between the Rejection and Quiescence cohorts. The Rejection cohort had higher serum creatinine levels and lower eGFR than the Quiescence cohort, as expected. No differences were observed in race, sex, type of transplant, time post-transplant, HLA class II mismatches, panel-reactive antibodies, induction therapy, or maintenance immunosuppression. This set of independent samples validated that the AlloMap Kidney classifier distinguished quiescence (median, 9.49; interquartile range [IQR], 7.68–11.53) from rejection (median, 13.09; IQR, 11.25–15.28; $P < 0.001$; Figure 1A). The medians (IQRs) for each of the sample groups were as follows: HS, 10.04 (8.38–11.85); pNR, 8.73 (7.45–11.13); NR, 10.07 (8.05–12.14);

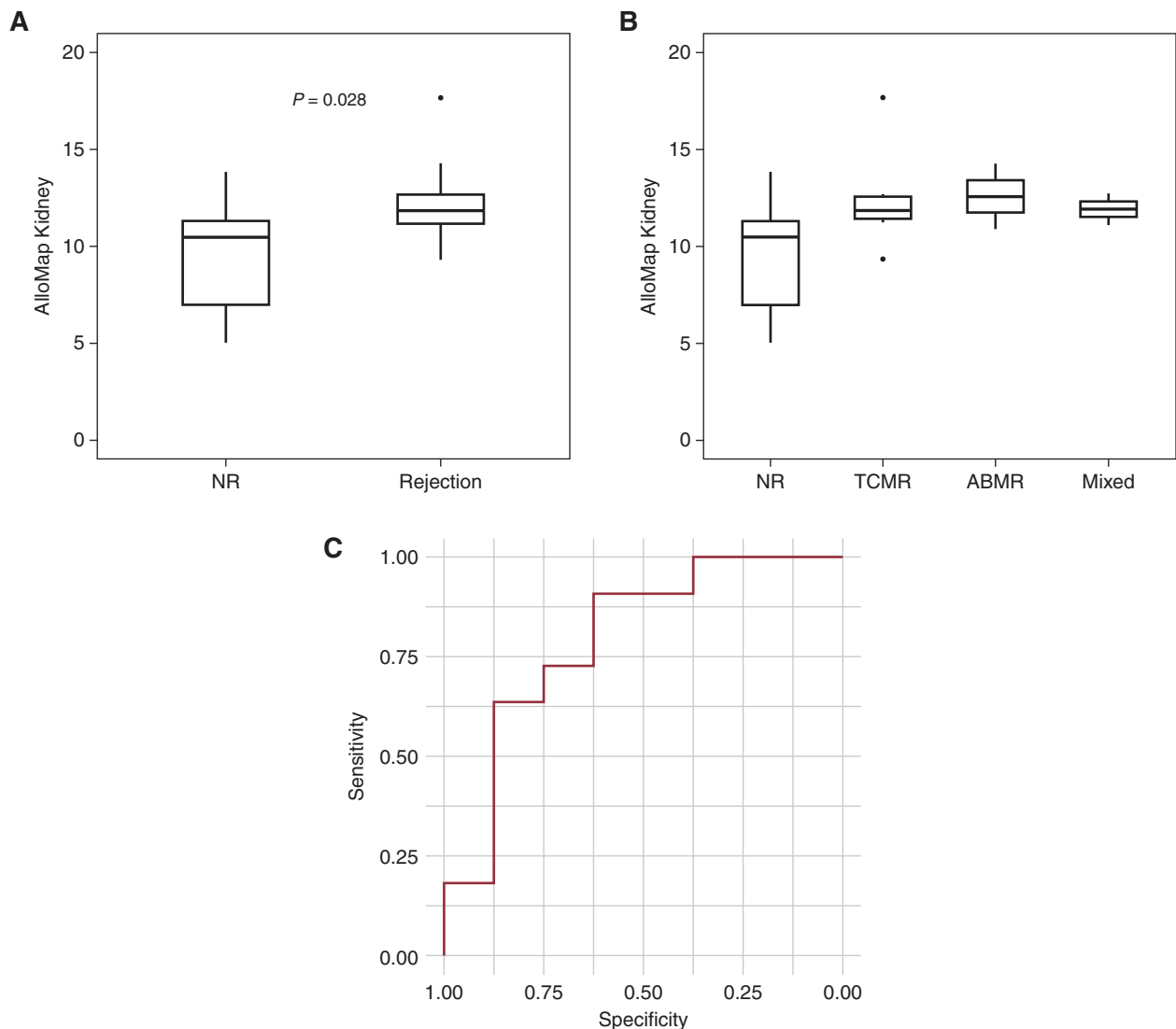


Figure 2. | AlloMap Kidney differentiates quiescence from rejection in a single-center validation set. (A) Biopsy specimen–defined rejection ($n=11$) is significantly different from biopsy specimen–defined no rejection ($n=8$). (B) All three rejection groups are elevated relative to NR (NR, $n=8$; TCMR, $n=7$; ABMR, $n=2$; mixed rejection, $n=2$). (C) Receiver operating characteristic plot for the second validation set NR versus rejection (TCMR, ABMR, and mixed rejection). Unpaired t test.

TCMR, 15.09 (11.99–17.42); ABMR, 11.48 (10.95–13.68), and mixed rejection, 14.33. Each of the three defined Quiescence groups was significantly different from the Rejection cohort (for rejection versus HS, $P=0.003$; for rejection versus pNR, $P<0.001$; for rejection versus NR, $P<0.001$; Figure 1B). Each of the defined types of rejection was different from the Quiescence cohort (Figure 1B), with $P=0.03$ and $P=0.001$ for ABMR and TCMR versus Quiescence, respectively. Although insufficient numbers were available for a robust analysis of the association of AlloMap Kidney scores with TCMR grade, the data suggest that higher grades of TCMR may have higher scores (Figure 1C). The AUC for quiescence versus rejection in the primary validation set was 0.786 (95% CI, 0.66 to 0.91), demonstrating the excellent performance of the GEP across the score range (Figure 1D).

To further validate the performance of AlloMap Kidney, a set of samples from a center not included in the DART

study was also evaluated. The Quiescence cohort in this set included eight NR samples, and the Rejection cohort contained 11 rejection samples (two ABMR, seven TCMR, two mixed rejection). In this set, AlloMap Kidney scores were significantly different between Quiescence (NR) and Rejection groups ($P=0.03$; Figure 2A). Both the TCMR and the ABMR samples had elevated scores relative to the NR group (Figure 2B). The TCMR group consisted of five IIA rejections and two borderline rejections, with a median (IQR) AlloMap Kidney score of 11.85 (11.26–12.67). The AUC for discriminating samples with rejection in this sample set was 0.796 (95% CI, 0.57 to 1; Figure 2C).

We also assessed the performance of AlloMap Kidney in the combined validation sets to provide an analysis with a larger number of rejection samples. In the combined analysis, the scores for the NR group (median, 10.19; IQR, 7.64–12.09) were significantly lower than the scores for the

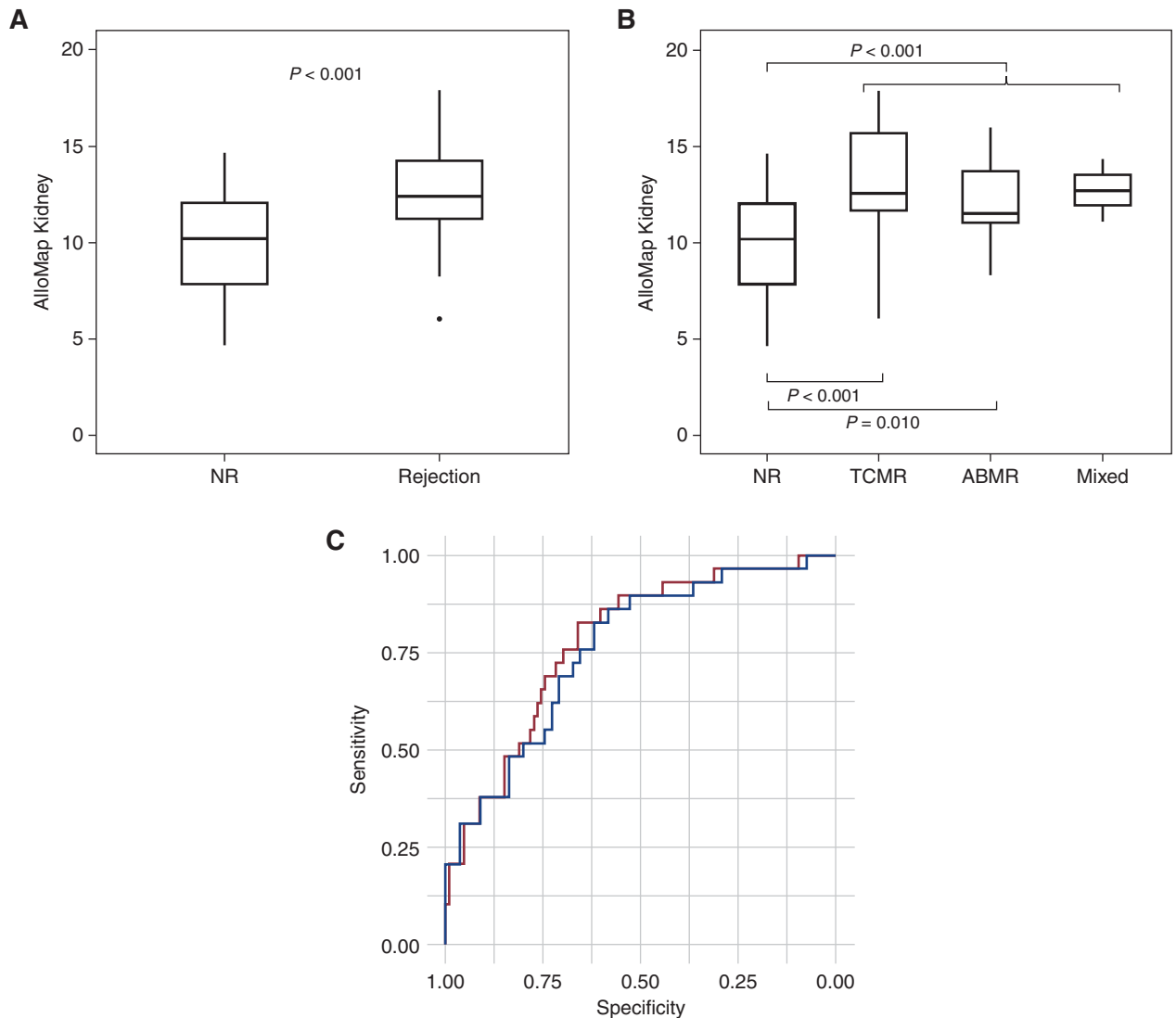


Figure 3. | The combined validation sets discriminate quiescence from rejection. (A) Quiescence ($n=55$) versus rejection ($n=29$) for the full combined datasets. (B) NR versus each type of rejection (NR, $n=55$; TCMR, $n=14$; ABMR, $n=12$, mixed rejection, $n=3$). (C) Receiver operating characteristic plot of the combined data. Red, quiescence ($n=106$) versus rejection, AUC=0.779; blue, NR versus rejection, AUC=0.776. Unpaired *t* test.

Rejection cohort (median, 12.43; IQR, 11.12–14.29); $P < 0.001$. All three Rejection groups showed elevated scores: for TCMR ($n=14$), median (IQR) of 12.55 (11.52–16.25); for ABMR ($n=12$), median (IQR) of 11.48 (10.95–14.06); and for mixed rejection ($n=3$), median (IQR) of 12.72 (11.12–14.33) (Figure 3B). Analysis of the combined independent validation sets resulted in an AUC of 0.779 (95% CI, 0.69 to 0.87) for quiescence versus rejection cohorts (Figure 3C).

NPV and positive predictive value (PPV) were determined at prevalence levels of 10% and 25%, representing the estimated prevalence of rejection on first-year surveillance and clinically indicated biopsies, respectively (4,9). The single-center sample set contained only NR in the Quiescent cohort; therefore, the performance of the classifier to differentiate the full quiescent group from biopsy sample-defined rejection was assessed on the DART validation set. Figure 4 shows the plots of the sensitivity,

specificity, PPV, and NPV of the classifier. For all performance metrics, the data are shown with either the full Quiescence cohort (HS, NR, and pNR) or with only the NR group. Sensitivity did not change because the Rejection cohort remained the same, but specificity, NPV, and PPV are dependent on the choice of quiescence cohort samples. The threshold used for this binary performance characterization was 11.5, at which the AlloMap Kidney score achieved the maximum accuracy for sensitivity and specificity (Figure 4). At the 11.5 score, AlloMap Kidney had a PPV of 23% and an NPV of 95% at 10% prevalence, and a PPV of 48% and an NPV of 87% at 25% prevalence to discriminate rejection from quiescence.

For samples in the DART study, plasma was also collected to measure dd-cfDNA using AlloSure. AlloSure is highly associated with graft injury (9); the signal is hypothesized to be different from that of the AlloMap Kidney

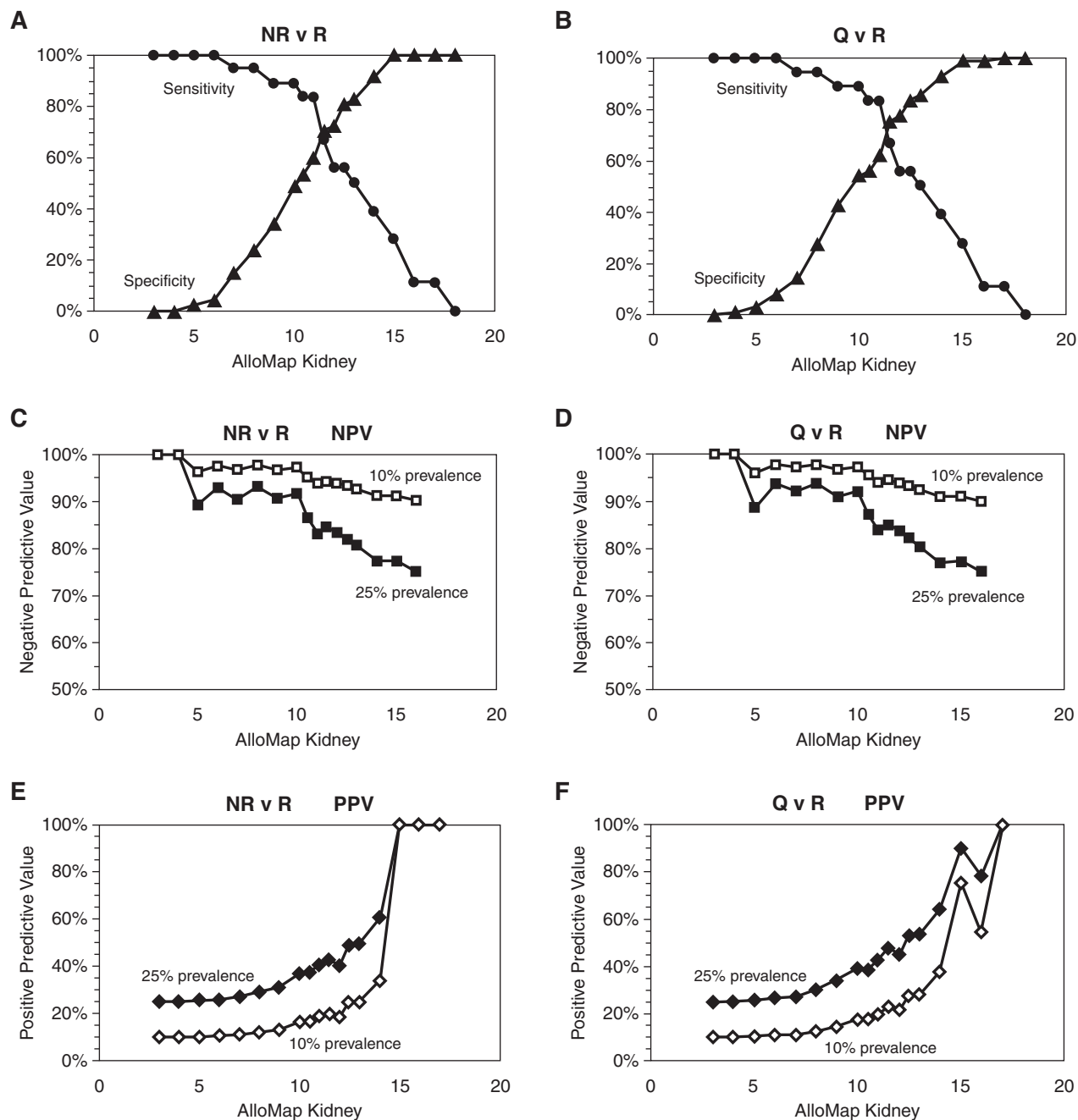


Figure 4. | AlloMap Kidney performance can be assessed across the range of scores. (A) Sensitivity (circles) and specificity (triangles) for NR versus rejection. (B) Sensitivity (circles) and specificity (triangles) for quiescence (Q) versus rejection (R). (C) Negative predictive value (NPV) for NR versus rejection. (D) NPV for quiescence versus rejection. (E) Positive predictive value (PPV) for NR versus rejection. (F) PPV for quiescence versus rejection. For NPV and PPV, 25% prevalence is shown in filled symbols, 10% prevalence in open symbols.

signature, although both are correlated with rejection. Figure 5A shows the data for all quiescence and rejection cohorts (mixed rejection was included in the ABMR group). There was a weak correlation between AlloSure and AlloMap Kidney ($R=0.15$, $P=0.23$). However, samples with AlloSure results of $\geq 1\%$ had higher AlloMap Kidney scores. Several TCMR samples with AlloSure between 0.5% and 1% had very high AlloMap Kidney scores, suggesting a role for AlloMap Kidney to inform on which of these

intermediate scores likely correlate with rejection (11). To examine the potential of coupled testing in post-transplant care, a combined score was derived with equal weighting of AlloSure and AlloMap Kidney. The range of this combined score can be envisioned along the diagonal from lower left in the plot in Figure 5A to the upper right. These data were used to generate an ROC plot for the combined score, which was compared to the ROC plots for AlloSure or AlloMap Kidney alone in the same sample set (Figure 5B). These data

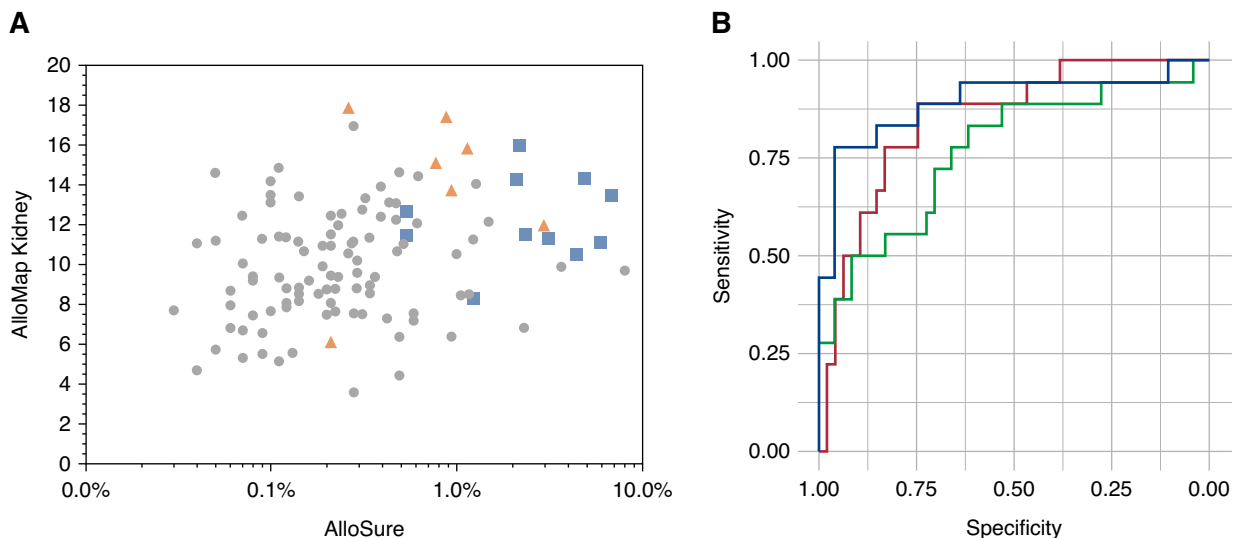


Figure 5. | The combination of AlloMap Kidney with AlloSure has greater discriminating ability than either alone. (A) Plot of all quiescence and rejection points from the validation set for both AlloMap Kidney (y axis) and AlloSure (x axis). Circles are all types of quiescence (NR, HS, pNR). Orange triangles, TCMR; blue squares, ABMR. (B) Receiver operating characteristic plots of a linear combination of AlloMap Kidney and AlloSure scores as shown in (A). Blue is the combined score (AUC=0.894), AlloMap Kidney alone is shown in green (AUC=0.768), and AlloSure alone is shown in red (AUC=0.85).

showed a superior performance for the combined use of AlloMap Kidney and AlloSure versus AlloMap Kidney alone ($P=0.005$).

Discussion

Factors predisposing the development of active rejection have been extensively studied, with the recipient immune system proving to be a key intermediary in many relevant processes, including ischemia-reperfusion injury, infection, and response to immunosuppression. Uncontrolled inflammation in kidney allografts leads to the chronic damage and progressive fibrosis that accounts for the majority of long-term allograft loss (28,29). Genetic predictors of active rejection have also been described in recent years, some of which implicate immune activity. Taken in concert, these data suggest that monitoring gene expression in peripheral blood immune cells may lead to earlier or more sensitive detection of active rejection (19,20,30).

The objective of this study was to validate a classifier that discriminates immune quiescence from kidney allograft rejection. Rather than novel discovery from the whole transcriptome, this classifier was developed using a candidate gene approach using the AlloMap Heart genes, which have been implicated in immune responses or regulation and, therefore, presented a rational starting point for developing a quiescence signature for kidney transplantation. Furthermore, we elected to measure whole blood gene transcripts rather than looking specifically at the PBMC subset, the method used by the AlloMap Heart assay. Collection of whole blood is significantly less complicated than PBMC collection, enabling a more streamlined workflow at the collection site. After accurately measuring the expression of these candidate genes, we identified five that could

discriminate kidney rejection using a training sample set from the DART study that was collected in PAXgene tubes. This classifier was tested using two validation sets: the primary validation set comprised independent patients from DART, and the second validation set was from a single center. Both sample sets demonstrate the validity of the classifier to discriminate biopsy sample-defined rejection from quiescence. Classifier scores were statistically significantly different between the rejection cohort and the quiescence cohort, and between the quiescence cohort and either TCMR or ABMR. Results in the three subgroups of quiescence (HS, pNR, NR) were each statistically different from those seen in rejection. In both validation sets, the AUC demonstrated excellent diagnostic performance. The classifier had an NPV of >95% in a surveillance population (10% prevalence) on the basis of a score of 11.5, chosen for maximal sensitivity and specificity.

The limitations of this study include the number of rejection samples and limited scope of the patient population. To generate training and validation sets from completely independent patients, we were limited to only 18 rejection samples in each set, randomly selected to generate the two independent sets. Despite these small numbers, independent validation sets of TCMR and ABMR, and of mixed rejection, demonstrated excellent diagnostic performance in all comparisons. Further, a second independent validation set recapitulated the performance of the classifier in the primary validation set. Although three different types of quiescence samples (NR, pNR, and HS) were included in this study, there may be other subsets of the target patient population that should be independently characterized on this classifier in future studies, such as infection, interstitial fibrosis and tubular atrophy, drug toxicity, BKV nephropathy, and recurrent or *de novo* glomerular disease. Each of these could have been undiagnosed in the HS set, which

serves as an indicator of a surveillance population at large. However, clinicians may still wish to biopsy to identify some of these pathologies in patients with high suspicion. The results presented here represent assessment of the status of the allograft at the time of the AlloMap Kidney testing, rather than long-term graft survival. A correlation with long-term outcomes necessarily requires large, prospective studies and does not detract from the demonstrated capability of AlloMap Kidney to provide noninvasive assessment of current rejection status. Lastly, our approach necessarily limited the scope of possible genes and, therefore, may not have included the best genes for discrimination of rejection from immune quiescence. With further sample availability from ongoing studies, we anticipate a robust sample set to potentially expand the clinical utility of the assay with expanded gene sets.

One strength of this study is that we started with a well-validated gene set and test condition. The gene set from the Food and Drug Administration–cleared AlloMap Heart test has proven robust and clinically relevant for >15 years in heart transplantation (17). In contrast, within kidney transplantation, previously developed gene expression profiling assays have limited overall use in more narrow clinical indications, such as replacing surveillance biopsies or assessment of fibrosis (19,21).

In contrast to the AlloMap Kidney measure of immune activity, the use of AlloSure to quantify dd-cfDNA provides insight into molecular injury in the allograft. Prior experience with combined testing in heart transplantation suggested that gene expression measurement in immune cells provides a complementary signal that can provide added insight into allograft rejection. Gene expression profiling may discriminate between types of allograft injury, including drug toxicity that can lead to injury in the absence of rejection. Therefore, there is potential added value when the two tests are combined. Indeed, when the DART validation set was analyzed using both AlloMap Kidney and AlloSure, a higher diagnostic performance was observed. Only a single rejection sample was below the nominal threshold for both tests. The details of the pathology report for this sample were independently reviewed and, despite being called TCMRIA by the pathologist at the treating center, the biopsy specimen only showed focal mild tubulitis. On the basis of Banff 2019, this biopsy specimen would not even meet the criteria for borderline TCMR; it would be called acute tubular injury on the basis of the pathology report. A *post hoc* reanalysis of the AlloMap Kidney AUC with this sample in the NR set produced a value of 0.83. Future studies will better define how these complementary signals can enhance diagnostic assessment and management of recipients of kidney transplants. In the primary validation cohort, AlloMap Kidney scores appear higher in TCMR than in ABMR, whereas AlloSure scores have been reported higher with ABMR (9). The coupled use of these assays may allow noninvasive discrimination of the type of rejection. The high NPV of AlloMap Kidney makes it an ideal assay for integration into routine post-transplant care, allowing minimization of biopsies in the same manner as AlloMap Heart has in heart transplantation for >15 years. This may be complemented by the AlloSure signal indicating injury, thus improving the PPV.

Future analysis of larger datasets of paired data will provide detailed performance of the use of both tests together.

Immune activity biomarkers can strengthen the high NPV of existing markers, allowing the confidence to rule out pathology by identifying those who are immunoquiescent. These types of markers also open the prospect of managing immunomodulation. Reducing medication dose for patients who are adequately immunosuppressed, and increasing the dose in those patients who are not, may lead to improved outcomes for both patient populations. The combination of allograft injury (AlloSure dd-cfDNA) and immune activation markers (AlloMap Kidney GEP) also take us in the direction of noninvasive characterization of underlying pathology and one step closer to offering a true liquid biopsy for monitoring allograft health. With uncontrolled inflammation playing such a fundamental role across the spectrum of allograft loss, noninvasive characterization of both injury and gene expression testing presents an attractive paradigm shift toward transplant precision medicine.

Disclosures

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Author Contributions

E. Akalin, D.C. Brennan, J.S. Bromberg, S. Dholakia, R.N. Woodward, and H. Xu conceptualized the study; E. Akalin, R.N. Woodward, and H. Xu provided supervision; S. Dholakia, X. Jin, and H. Xu were responsible for validation; S. Dholakia and R.N. Woodward wrote the original draft; X. Jin, R.N. Woodward, and H. Xu were responsible for data curation and methodology; X. Jin and H. Xu were responsible for investigation; R.N. Woodward was responsible for resources; R.N. Woodward and H. Xu were responsible for formal analysis and project administration; and all authors reviewed and edited the manuscript.

Supplemental Material

This article contains the following supplemental material online at <http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0005062021/-/DCSupplemental>.

Supplemental Appendix 1. Supplemental methods.

Supplemental Figure 1. Conversion of AlloMap Kidney classifier trained on qPCR data to produce the same results from RNA-seq data.

Supplemental Figure 2. Comparison of AlloMap Kidney data from manual and automated extraction methods.

Supplemental Table 1. Overview of the AlloMap genes.

Supplemental Table 2. List of the AlloMap reference genes.

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